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Enzymatic Screening and Characterization of Cellulolytic Actinomycetes Isolated from Soil samples collected from Patna District in Bihar, India

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KEYWORDS

Actinomycetes, Cellulolytic strains, Physico-chemical parametes, CMC-ase, FP-ase

A B S T R A C T

In the present study, 10 cellulolytic actinomycete strains were isolated from soil samples collected locally from Patna region in Bihar, India. The strains were grown at different temperatures, pH, non-ISP media, ISP media and carbohydrates for optimization of physico-chemical parameters for the selected strains. All the strains except CD-2 grew well between temperatures 26 and 50°C and pH 7 and 9. The strains were able to grow on all the specified non-ISP media, ISP media and carbohydrates but the most preferred one was Starch casein Agar, ISP-4 and Dextrose. Assessment of cellulolytic potential of the selected strains showed that the strains CD-3 (0.023 IU/ ml CMCase unit and 0.009 IU/ ml FPase unit) and CD-10 (0.020 IU/ ml CMCase unit and 0.010 IU/ ml FPase unit) were the most efficient cellulose degrading actinomycete strains.

Introduction

Sustainable resources, which are the need of present time, can be derived from plant biomass. Photosynthesis ensures perpetual renewal of plant biomass and thus an inexhaustible supply of such material. By means of chemical or bioconversion methods, cellulosic biomass can be converted into food, fuel and chemicals (Couglan, 1985). Since the microorganisms are characterized by their rapid growth, therefore, bioconversion by cellulolytic microorganisms which is controlled and processed by the enzymes of cellulase system seems to be an economical and eco-friendly option. The cellulases include endo-acting CMC-ase (endoglucanases, EGs) and exo-acting FP-ase (cellobiohydrolases, CBH) enzymes, which act in a synergistic manner in biomass-degrading microbes (Dashtban *et al.*, 2009). Actinomycetes form an important part of the microbial community responsible for cellulose degradation (Vinogradova and Kushnir, 2003). In addition, their growth as branching hyphae is well adapted to the penetration and aid in the degradation of lignocellulose. Actinomycete cellulases are inducible extracellular enzymes (Ibrahim and El-diwany, 2007) that can be produced during their growth on cellulosic materials.

Cellulase production in cultures is growth associated and is influenced by various factors including pH, temperature; growth media and their interactions can affect cellulase productivity (Tholudur *et al.*, 1999). Successful bioconversion of cellulosic materials mainly depends on the nature of cellulose, sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes (Alam *et al.*, 2004). According to Van Zyl (1985), optimization of fermentation media ingredients and environmental factors for enzyme production is a more convenient and effective strategy.

The present work, therefore, concentrates on enzymatic screening and characterization of actinomycetes isolated from soil samples collected from Patna District in Bihar, India.

Materials and methods

Collection of soil samples

The soil samples were collected locally from a depth of 3–4 inches from an agricultural field, municipal dumps and domestic wastes in Patna region. The collected soil samples were kept in polythene bags and brought to the laboratory for analysis.

Chemicals: Most of the chemicals used for the preparation of the media were obtained from HiMedia, LOBA CHEMIE, MERCK and QUALIGENS.

Media: Like all the other living organisms, the microorganisms also need certain basic nutrients for the sustenance of life. The media provide the nutritional requirements of the microbial cells in the laboratory. Different media were utilized for the isolation, preservation, enzymatic screening, biochemical and cultural characterization of the cellulose degrading actinomycetes strains.

Medium for isolation and preservation of actinomycetes

Starch Casein Agar (Soluble starch 10 g/l, Casein 0.3 g/l, K_2HPO_4 2 g/l, $CaCO_3$ 0.02 g/l, $FeSO_47H_2O$ 0.01 g/l, KNO_3 2 g/l, $MgSO_47H_2O$ 0.05 g/l, NaCl 2 g/l, Agar 18 g/l) (Dubey and Maheshwari, 2004).

Medium for enzymatic screening

CMC Agar (carboxymethylcellulose 0.5 g/l, $NaNO_3$ 0.1 g/l, K_2HPO_4 0.1g/l, $MgSO_4$ 0.05g/l, yeast extract 0.05g/l, agar 15 g/l) (Kasana *et al.*, 2008).

Media for biochemical tests / Reagents

The composition of media and reagents meant for Indole test, methyl red test, Voges-Proskauer test, citrate utilization test, catalase test, urease test, starch hydrolysis test, gelatin hydrolysis test, sugar fermentation test, caseinase test, hydrogen sulphide production test and nitrate reduction test were noted down from Practical Microbiology by Dubey and Maheshwari (2004).

Other growth media for cultural characterization of strains

Non-ISP media included Cellulose Congo Red Agar (K_2HPO_4 0.50 g/l, $MgSO_4$ 0.25 g/l, Cellulose powder 1.88 g/l, Congo red 0.20 g/l, Agar 15 g/l, Gelatine 2 g/l); Mcbeth medium (K_2HPO_4 1 g/l, $CaCO_3$ 2 g/l, Na_2SO_4 2 g/l, $MgSO_4.7H_2O$ 1 g/l, $[(NH_4)_2SO_4]$ 2 g/l, CMC 1.0 %, Agar 15 g/l); Stanier's basal medium $[(NH_4)_2SO_4$ 1g/l, K_2HPO_4 1 g/l, $MgSO_4$ 0.2 g/l, $CaCl_2$ 0.1 g/l, $FeCl_3$ 0.02 g/l, Agar 15 g/l]; Starch Casein Agar (previously mentioned) and Nutrient Agar (Peptone 5g/l, NaCl 5g/l, Beef extract 3g/l, Agar 15g/l) for cultural characterization of strains. ISP media

included ISP 1, ISP 2, ISP 3, ISP 4, ISP 5, ISP 6 and ISP 7 for the above purpose. Different carbon sources including 0.5% (w/v) of each of sucrose, lactose, dextrose, fructose, mannitol and inositol incorporated separately in mineral salt medium for studying the capability of the cultures to utilize different carbon sources as source of energy.

Isolation and purification of the actinomycetes strains from the collected soil samples

The soil samples were serially diluted in sterilized normal saline and the aliquots of fivefold of the soil solutions were plated on the sterilized solidified Starch Casein Agar medium in the petriplates in aseptic condition. The plates were incubated at 26°C for 4 days. The different colonies of actinomycetes thus obtained were purified by single streak method and screened for their cellulolytic activities.

Screening of cellulose degrading microorganisms

The pure actinomycetes cultures were allowed to grow on CMC Agar plates at 26°C for 7 days. The microbial colonies were screened for the enzyme production by flooding the plates with 0.1 % Congo red for 15 min followed by counterstaining with 1N HCl for another 15 min. The solubilization of cellulose is indicated by clear zones around the colonies. Those strains, showing clearing or solubilizaton zones, were presumed to be cellulose degraders, and were selected for further investigation in terms of quantitative analysis.

Morphological characterization of the selected strains

The strains were macro-morphologically characterized by observing the color, texture and pigmentation of the culture colonies on Starch Casein Agar medium after 4 days of incubation at 26°C and micro-morphologically by preparing slides of the fresh cultures aseptically.

Optimization of environmental and cultural conditions for the selected strains

Physical conditions like the temperature and the pH of the extracellular environment greatly affects the growth and enzymatic activities of the microorganisms. The strains were placed on Starch Casein Agar and incubated at temperatures 4, 26, 37, 45 and 60°C for 4 days and the growth was recorded. For optimization of pH, the strains were plated separately on five sets of Starch Casein Agar plates having pH 3, 5, 7, 9 and 11 and were incubated at 26°C for 4 days and the growth was recorded. The strains were grown on different non-ISP, ISP media and carbon sources at 26°C for 4 days in order to investigate their adaptability to different growth media and also to find the best suited growth medium for cultivation of the cultures.

Assessing cellulolytic potential of the selected strains

Four days old culture broths of the strains were centrifuged at 5000 rpm for 15 min and the supernatant was used as crude enzyme for cellulase assay. The cellulose degrading capacity of the selected microorganisms was quantified by estimating the amount of reducing sugar released during cellulose degradation by DNS method. CMC-ase activity and FP-ase activity of the strains were assayed using the methods recommended in IUPAC (Ghose, 1987) with slight modifications. For CMC-ase activity, 0.5 ml of enzyme solution was added to 0.5 ml of 1% (w/v) of

CMC prepared in sodium citrate buffer (pH 6.0) in a test tube and incubated at 50°C for 60 min. For FP-ase activity, 0.5 mL of enzyme solution was added to 0.5 mL of sodium citrate buffer (pH 6.0) along with 50 mg Whatman No. 1 filter paper strip (1x6 cm) in a test tube and incubated at 50°C for 60 min. In both the above mentioned procedures, the reactions were stopped by adding 1.0 mL 3, 5-dinitro salicylic acid reagent to the mixtures followed by boiling for 10 min and then cooling. The optical density (O.D.) of the mixtures was recorded at 540 nm using UV/Vis spectrophotometer (Thermo Scientific) and compared with the standard glucose curve to determine the amount of reducing sugar (mg mL-1) produced during cellulose hydrolysis by DNS method (Miller, 1959). The CMC-ase and FP-ase activities were defined in International Units (IU). One unit of enzymatic activity is defined as the amount of enzyme that releases 1 μmol reducing sugar (measured as glucose) per ml per minute.

Results and discussion

Isolation and purification of the actinomycetes strains from the collected soil samples

Thirty five actinomycetes colonies were obtained on Starch Casein Agar plates by plating the aliquots of fivefold serially diluted soil solution. The colonies were purified by single streak method on Starch Casein Agar plates.

Screening of cellulose degrading microorganisms

After preliminary screening by Congo red test, ten strains labeled as CD-1, CD-2, CD-3, CD-4, CD-5, CD-6, CD-7, CD-8, CD-9 and CD-10 (Fig. 1) were selected for further investigation on the basis of area of clear zone around the colonies. Screening for the CMC-ase activity is based on the fact that β-1, 4-glucan interacts specifically with the dye Congo red to give a visible red shift (Teather and Wood, 1982).

Morphological characterization of the selected strains

Macroscopic observations of the colonies of the selected strains revealed their colonies to be of different color and powdery in texture (Fig. 2). On Gram staining and microscopic view, all the isolated strains were found to be Gram positive filaments suggesting them to be actinomycetes strains (Fig. 3).

Optimization of environmental and cultural conditions for the selected strains

One of the important factors of increasing the enzyme production in microorganisms is optimization of environmental conditions and medium compositions (Prasetsan and Doelle, 1987). The strains were grown at the specified temperatures and pH for optimization of physico-chemical condition for growth. The strains showed moderate to luxuriant growth between 26 and 45°C suggesting them to be thermotolerant. The strain CD-2, however, showed poor growth on 4th day of incubation indicating it to be a slow grower. The growth of all the strains was nil at the two specified extreme temperatures i.e. 4 and 60°C (Table 1). All the selected strains showed luxuriant growth on neutral to alkaline medium. These findings are similar to that of Solingen *et al.* (2001) who reported optimum pH of 8 for a novel *Streptomyces* species. Researchers have shown that the production costs of cellulase are tightly associated with the productivity of enzyme producing microbial

strains (Duff and Murray, 1996). The strain CD-2 being a slow grower, showed moderate to poor growth on 4th day of incubation. None of the strains, however, were able to grow in acidic medium as shown in Table 2. Van Zyl (1985) emphasized on optimization of fermentation media ingredients for enzyme production rather than using molecular techniques to synthesis endoglucanase. The strains were grown on the specified non-ISP, ISP media and carbon sources at 26°C for 4 days to find out the most suitable fermentation media. The observations are recorded in Tables 3–5, respectively. It was observed that the strains showed different degree of growth on all the specified non-ISP media and ISP media but the most preferred one was Starch casein Agar and ISP-4, respectively. Among the carbohydrates, all the strains grew on Dextrose but 60% of the strains preferred mannitol.

Assessing cellulolytic potential of the selected strains

The CMC-ase and FP-ase activities of the selected strains were determined by estimating the amount of reducing sugar (µg/ ml) released by them. The CMC-ase and FP-ase activities of the selected strains were determined by estimating the amount of reducing sugar (µg/ ml) released by them. It was investigated that the amount of reducing sugar released due to CMC-ase activity by the strains CD-1 and CD-9 was 48 µg/ ml; by the strain CD-2 was 42 µg/ ml; by the strain CD-3 was 63 µg/ ml; by the strains CD-4 and CD-5 was 51 µg/ ml; by the strain CD-6 was 47 µg/ ml; by the strain CD-7 was 45 µg/ ml; by the strain CD-8 was 50 µg/ ml and by the strain CD-10 was 56 µg/ ml, respectively. The amount of reducing sugar released due to FP-ase activity by the strains CD-1 and CD-3 was 49 µg/ ml; by the strain CD-2 was 50 µg/ ml; by the strains CD-4 and CD-5 was 48 µg/ ml; by the strain CD-6 was 54 µg/ ml; by the strains CD-7 and CD-8 was 52 µg/ ml; by the strain CD-9 was 53 µg/ ml and by the strain CD-10 was 55 µg/ ml, respectively. The data are presented in the Figure 4.

Table.1 Effect of temperature on growth of strains on 4th day of incubation

Strain	4°C	26°C	37°C	45°C	60°C
CD-1	-	+++	+++	+++	-
CD-2	-	+	+	+	-
CD-3	-	+++	+++	+++	-
CD-4	-	+++	+++	+++	-
CD-5	-	+++	+++	+++	-
CD-6	-	+++	+++	+++	-
CD-7	-	++	+++	+++	-
CD-8	-	+++	+++	+++	-
CD-9	-	+++	+++	+++	-
CD-10	-	+++	+++	+++	-

- No growth; + poor growth; ++ moderate growth; +++ luxuriant growth

Table.2 Effect of pH on growth of strains on 4th day of incubation

Strain	pH 5	pH 7	pH 9	pH 11
CD-1	-	+++	+++	+++
CD-2	-	+	++	+
CD-3	-	+++	+++	+++
CD-4	-	+++	+++	+++
CD-5	-	+++	+++	+++
CD-6	-	+++	+++	+++
CD-7	-	+++	+++	++
CD-8	-	+++	+++	+++
CD-9	-	+++	+++	+++
CD-10	-	+++	+++	+++

- No growth; + poor growth; ++ moderate growth; +++ luxuriant growth

Table.3 Growth of strains on different non-ISP media on 4th day of incubation

Strain	Cellulose Congo Red Agar	Mcbeth medium	Stanier's basal medium	Starch Casein Agar	Nutrient Agar
CD-1	++	+++	-	+++	++
CD-2	++	++	++	+++	+++
CD-3	++	+++	+	+++	+++
CD-4	+	++	++	+++	++
CD-5	+++	++	+	+++	+++
CD-6	+++	+	+	+++	+++
CD-7	+++	++	++	+++	+++
CD-8	+++	+++	++	+++	+++
CD-9	+++	++	+	+++	+++
CD-10	+++	+	-	+++	+++

- No growth; + poor growth; ++ moderate growth; +++ luxuriant growth

Table.4 Growth of strains on different ISP media on 4th day of incubation

Strain	ISP 1	ISP 2	ISP 3	ISP 4	ISP 5	ISP 6	ISP 7
CD-1	+++	-	-	+++	+	+	+++
CD-2	+++	+	-	++	+	+++	++
CD-3	+++	+	-	++	+	++	+++
CD-4	+	+	-	++	-	++	+
CD-5	+++	+++	+++	+++	-	++	++
CD-6	+++	++	+++	+++	++	++	++
CD-7	+	+++	++	+++	-	++	++
CD-8	+++	+++	+++	+++	+++	+++	+++
CD-9	+++	+++	+++	+++	+	+++	+++
CD-10	+++	++	+	+++	-	++	+++

- No growth; + poor growth; ++ moderate growth; +++ luxuriant growth

Table.5 Growth of strains on different carbohydrates on 4th day of incubation

Strain	Dextrose	Sucrose	Fructose	Lactose	Mannitol	Inositol
CD-1	++	+	++	+	++	++
CD-2	++	++	-	+	+	+
CD-3	+	+++	++	+++	+	++
CD-4	+	++	-	++	-	++
CD-5	+++	+++	+++	+	+++	+++
CD-6	++	++	++	+	++	++
CD-7	+	++	+	-	++	-
CD-8	+++	+	+	+	+++	+
CD-9	+	+++	++	++	+++	++
CD-10	+	-	+	+	+++	+++

- No growth; + poor growth; ++ moderate growth; +++ luxuriant growth

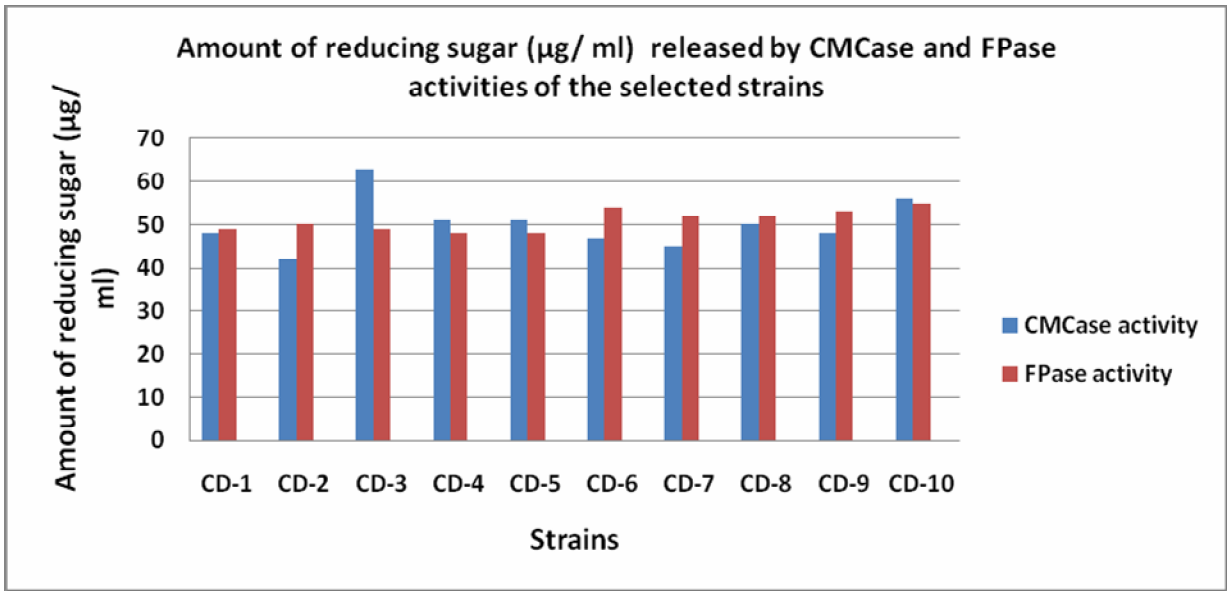

Fig. 4 CMC-ase and FP-ase activities of the selected strains in terms of the amount of reducing sugar released

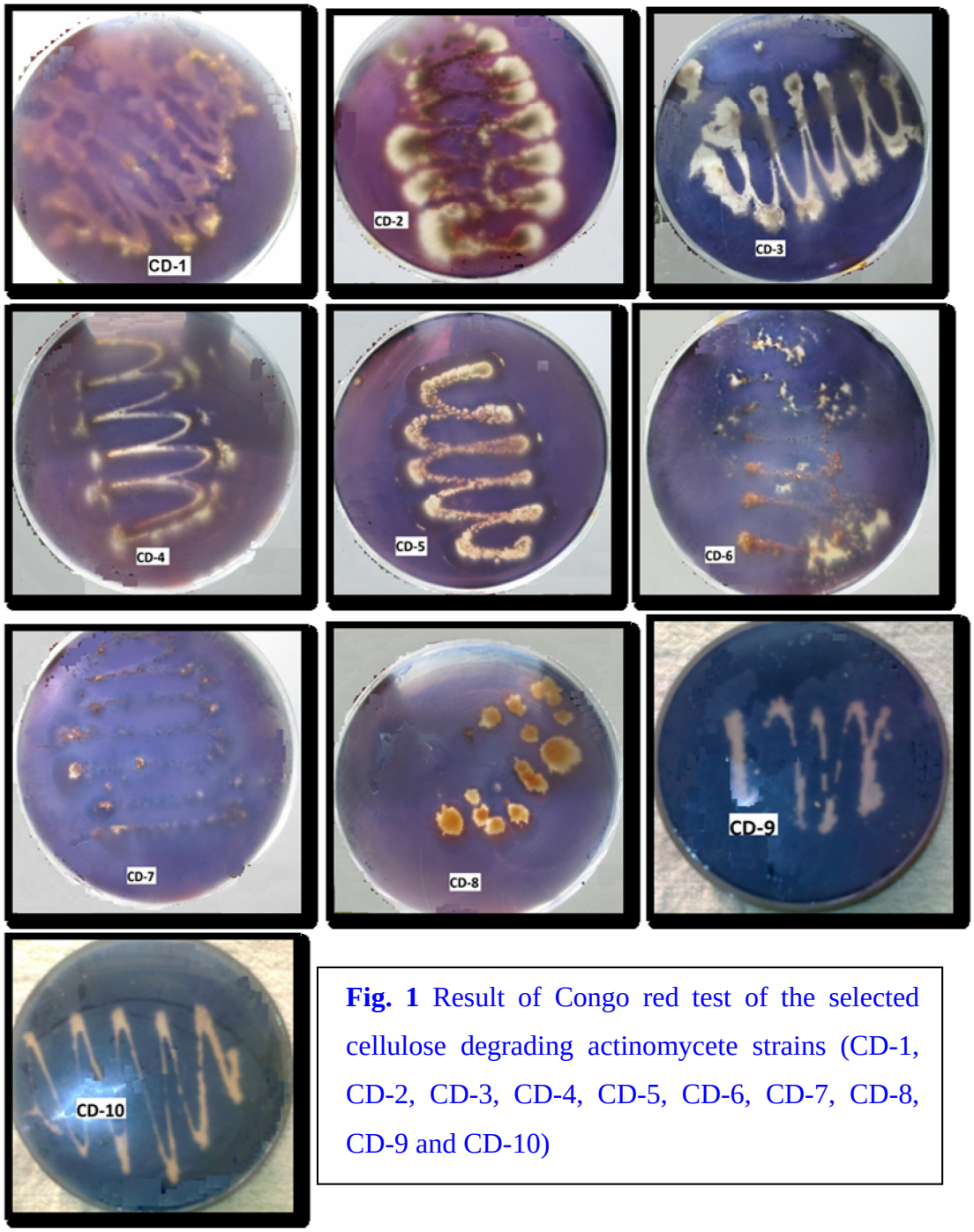

Fig. 1 Result of Congo red test of the selected cellulose degrading actinomycete strains (CD-1, CD-2, CD-3, CD-4, CD-5, CD-6, CD-7, CD-8, CD-9 and CD-10)

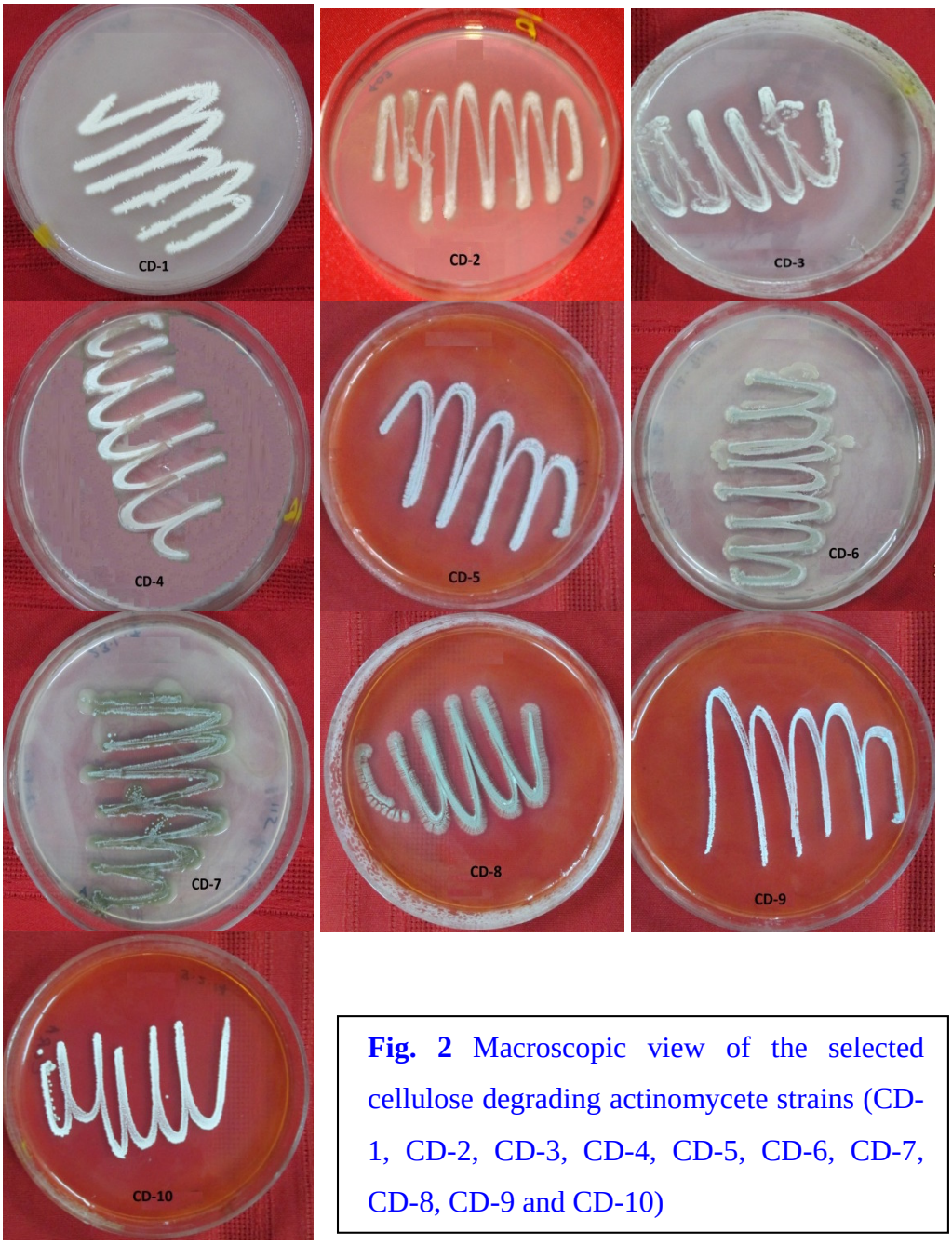

Fig. 2 Macroscopic view of the selected cellulose degrading actinomycete strains (CD-1, CD-2, CD-3, CD-4, CD-5, CD-6, CD-7, CD-8, CD-9 and CD-10)

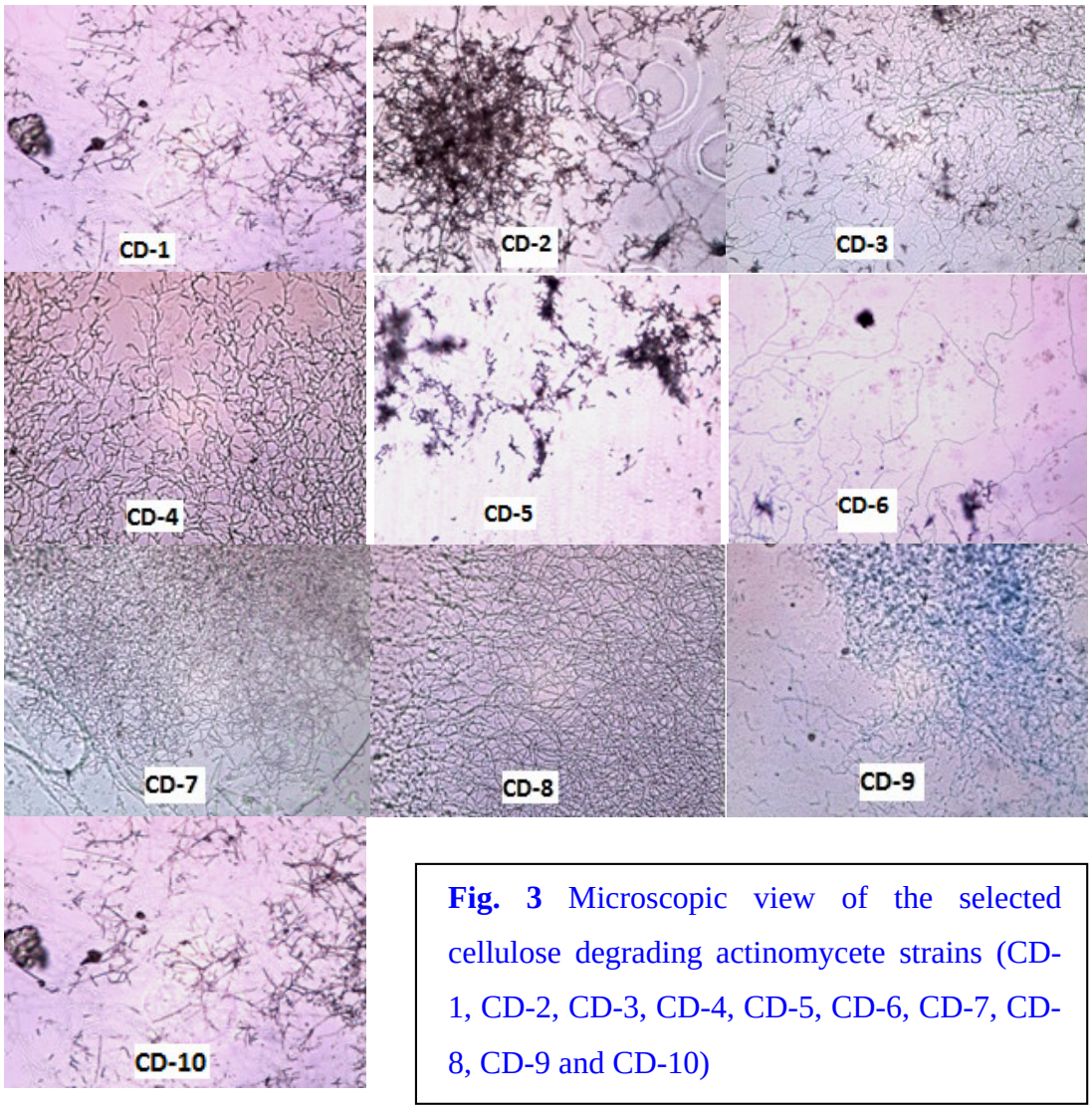

Fig. 3 Microscopic view of the selected cellulose degrading actinomycete strains (CD-1, CD-2, CD-3, CD-4, CD-5, CD-6, CD-7, CD-8, CD-9 and CD-10)

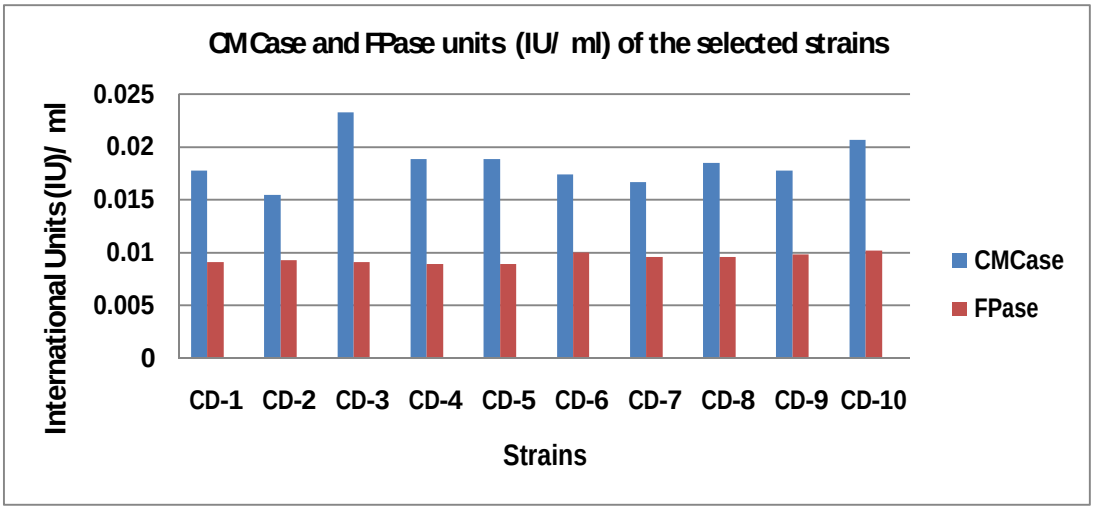

Fig. 5 CMC-ase and FP-ase activities of the selected strains in terms of International Units (IU) per ml

The CMC-ase and FP-ase activities of the selected strains were also defined in International Units (IU). It was investigated that the strains CD-1, CD-6 and CD-9 produced 0.017 IU/ ml of CMC-ase enzyme; the strain CD-2 produced 0.015 IU/ ml; the strain CD-3 produced 0.023; CD-4, CD-5 and CD-8 produced 0.018 IU/ ml; CD-7 produced 0.016 IU/ ml and CD-10 produced 0.020 IU/ ml of CMC-ase enzyme, respectively. The strains CD-1, CD-2, CD-3, CD-7, CD-8 and CD-9 produced 0.009 IU/ ml of FP-ase enzyme whereas the strains CD-4 and CD-5 produced 0.008 IU/ ml and the strains CD-6 and CD-10 produced 0.010 IU/ ml of FP-ase enzyme, respectively. The data are presented in Figure 5.

All the cellulase producing actinomycetes strains isolated in the present study have potential cellulolytic activity. The present investigation revealed that most of the strains were capable of exhibiting luxuriant growth at a wide range of temperature and pH and they were capable of utilizing a wide range of culture media and carbon sources. These features would help them survive in the fluctuating environmental conditions prevalent in India. Both CMC-ase and FP-ase enzymes act in a synergistic manner in biomass-degrading microbes and therefore, the strains CD-3 and CD-10 appear to be the most efficient cellulose degrading actinomycete strains among all the studied actinomycete strains. These strains can be further exploited to be commercially used for the purpose of cellulose hydrolysis and production of useful products.

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